TITLE **Defence against antimicrobial peptides: different strategies in Firmicutes**

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RUNNING TITLE Antimicrobial peptide resistance in Firmicutes

Summary

The Firmicutes constitute a phylum of bacteria that can be found in a wide variety of habitats, from soil to the gastrointestinal tract of animals, where they have to thrive in complex communities. Competition in these communities usually involves the production of compounds such as antimicrobial peptides to eliminate competitor organisms. Animals and plants also produce antimicrobial peptides to control their associated microbiota. In turn, defence mechanisms have evolved to prevent the action of these compounds. The close association of some Firmicutes with humans as prominent pathogens or commensal organisms has driven a considerable research effort on defence mechanisms used by these bacteria against antimicrobial compounds. This review focuses on the most recent advances on two well characterized defence mechanisms against antimicrobial peptides: the modification of the cell wall by D-alanylation and the role of peptide antibiotic-specific ABC transporters.

Introduction

Antimicrobial peptides (AMPs) are a diverse group of compounds produced by bacteria as well as higher organisms, including animals and humans. Among the most prominent examples of bacterial AMPs are the heavily modified lantibiotics, a class of bacteriocins that were named after their characteristic lanthionine or methyllanthionine residues. Their structure can be either elongated, for example in nisin (Fig. 1A) or subtilin, or globular, as is the case for mersacidin or actagardine (Bierbaum and Sahl, 2009). Non-lantibiotic bacteriocins are similar in size to lantibiotics (<10 kDa), but are not as extensively modified (Cotter et al., 2005). Both classes of bacteriocins are ribosomally synthesized and mainly produced by Firmicutes bacteria. Gram-negative bacteria also produce AMPs, usually referred to as microcins. These are ribosomally synthesized, essentially hydrophobic peptides that in some cases are subjected to post-translational modifications (Rebuffat, 2012). In addition, many bacteria also produce non-ribosomally synthesized peptides such as the small circular metallo-peptide bacitracin (Fig.

1A) (Johnson et al., 1945; Economou et al., 2013), or lipodepsipeptides such as ramoplanin (Fig. 1A) or enduracidin (Fang et al., 2006). AMPs also comprise a component of the innate immune system of higher organisms such as protegrins (Fig. 1A). Another example of mammalian AMPs are the defensins, which are produced for example in epithelial and immune cells of humans. They are about 30-40 amino acids long cysteine-rich peptides and adopt a conformation stabilized by disulfide bridges (Yount and Yeaman, 2013). With the exception of microcins, the examples mentioned above share a predominant positive charge and are therefore also referred to as cationic AMPs (CAMPs). Many more examples exist and have been extensively reviewed elsewhere (see for example (Breukink and de Kruijff, 2006; Nguyen et al., 2011; Yount and Yeaman, 2013).

AMPs inhibit bacterial growth, either to provide a competitive advantage to the producer in mixed bacterial populations or as a host defence mechanism against pathogens of higher organisms. To combat this, the targeted bacteria have to be able to detect and respond to AMPs in their environment. The sensitivity and efficiency of these processes are important factors for the survival of bacteria in competitive habitats such as the soil or the intestinal tract of mammals.

The primary mode-of-action of the AMPs addressed in this review is the inhibition of cell wall synthesis (Fig. 1B), although additional activities have been described for some compounds, e.g. pore-formation by nisin-type lantibiotics (Schneider and Sahl, 2010; Scherer et al., 2013) or disturbance of membrane function by bacitracin (Ming and Epperson, 2002; Schneider and Sahl, 2010; Economou et al., 2013). Details of the synthesis of the peptidoglycan polymer that constitutes the bacterial cell wall have been reviewed elsewhere (van Heijenoort, 2007; Bouhss et al., 2008). In brief, the biosynthetic cycle is initiated on the cytoplasmic side by assembly of precursor molecules and their attachment to the lipid carrier undecaprenyl-phosphate (UP; Fig. 1B). The resulting complex of N-acetylglucosamine-N-acetylmuramyl-pentapeptide, covalently coupled to the lipid carrier via a pyrophosphate linker is referred to

as lipid II (Fig. 1B) (van Heijenoort, 2007). After flipping of lipid II to the outer face of the cytoplasmic membrane (Mohammadi et al., 2011), the peptidoglycan subunits are incorporated into the growing cell wall. This step is the target for many CAMPs (Fig. 1B), e.g. the lantibiotics, which bind to the pyrophosphate moiety of lipid II on the outer face of the membrane (Bonev et al., 2004; Hsu et al., 2004).

After removal of the peptidoglycan precursors, the lipid carrier remains in the pyrophosphate form (UPP), which is dephosphorylated by UPP-phosphatases (Bouhss et al., 2008) and flipped back to the cytoplasmic face of the membrane (Fig. 1B). This recycling step is inhibited by bacitracin, which tightly binds to the pyrophosphate group and thus prevents the dephosphorylation reaction (Fig. 1B) (Siewert and Strominger, 1967; Storm and Strominger, 1973; Schneider and Sahl, 2010; Economou et al., 2013). CAMPs therefore appear to act as competitive inhibitors of cell wall synthetic enzymes, which is in contrast to the irreversible inactivation of penicillin binding proteins by the paradigmatic cell wall-active β-lactam antibiotics (Fisher et al., 2005) and is an important factor to consider when studying CAMP resistance.

To counteract CAMP action, bacteria have developed a broad range of resistance mechanisms, which include drug-specific responses such as proteolytic degradation (Sun et al., 2009) or increased production of the inhibited enzyme (Cao and Helmann, 2002), as well as less specific strategies such as biofilm formation (Otto, 2006). This review will concentrate on two major and widely distributed resistance mechanisms employed by Firmicutes to counteract CAMPs that have recently gained a significant amount of attention: changes in cell surface charge and AMP detoxification by transporters.

One way of relieving inhibition by CAMPs competing for substrate binding is to reduce the access of the peptides to the surface of the cytoplasmic membrane, i.e. the location of its target molecules. The best understood mechanisms to achieve this are the D-alanylation of teichoic acids, catalyzed by the DltABCD system (Perego et al., 1995; Neuhaus and Baddiley,

2003; McBride and Sonenshein, 2011; Reichmann et al., 2013), and the lysinylation of membrane phospholipids by MprF (Peschel et al., 2001; Oku et al., 2004; Andrä et al., 2011). For example, the sensitivity of a *dltA* mutant strain of *Lactobacillus casei* BL23 increased 12.5-fold for nisin, 4.25-fold for vancomycin, 16-fold for plectasin, 4-fold for mersacidin and 2.5-fold for subtilin, relative to the wild type strain (Revilla-Guarinos et al., 2013). In turn, inactivation of MprF in *Staphylococcus aureus* Sa113 resulted in a 28-fold increased sensitivity for nisin, 7-fold for gallidermin or 12-fold for protegrin 3 (Peschel et al., 2001). Both mechanisms are thought to reduce the net negative charge of the cell envelope, thus decreasing electrostatic interactions between CAMPs and the cell (Fig. 2B). Recently, a second mode of action of the Dlt-system was proposed, based on steric hindrance of CAMP passage through the cell wall due to an increased density of the peptidoglycan sacculus (Fig. 2C) (Saar-Dover et al., 2012).

A further mechanism of CAMP resistance is by antibiotic-specific ATP-binding cassette (ABC) transporters (Fig. 2), which are thought to remove the peptides from their site of action. A number of these transporters have been described. For example, the BceAB system of *Bacillus subtilis* confers resistance to bacitracin. A 143-fold increased sensitivity in *B. subtilis* 168 BceAB defective mutants has been reported (Ohki et al., 2003). Inactivation of the homologous system ABC 09 of *L. casei* BL23 resulted in an increased sensitivity to bacitracin (2-fold), nisin (1.7-fold) plectasin (2-fold) and subtilin (2.5-fold) relative to the wild type strain (Revilla-Guarinos et al., 2013). However, in contrast to canonical drug efflux systems for antibiotics that target intracellular structures, it is less obvious to envision how a transporter could impart efficient resistance against a drug that binds molecules located on the surface of the cell. For some CAMP transporters, a mechanism akin to the “hydrophobic vacuum cleaner” model has been proposed involving translocation of the peptide from the membrane to the culture supernatant (Stein et al., 2003; Stein et al., 2005; Okuda et al., 2008). The different types of CAMP transporters and their proposed functions are covered in detail below.

CAMP resistance by D-alanylation of teichoic acids: electrostatic or steric hindrance?

The cell wall of Gram-positive bacteria essentially consists of several layers of peptidoglycan interwoven with additional glycopolymers such as teichoic acids (Neuhaus and Baddiley, 2003). Structure and function of teichoic acids has been the subject of a number of excellent reviews (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008; Silhavy et al., 2010; Swoboda et al., 2010), and they will not be discussed in detail. Briefly, TAs are linear polymers typically constituted by monomers of glycerol-P or ribitol-P linked by phosphodiester bonds. These polymers can be attached to the peptidoglycan (wall teichoic acids, WTAs) by a glycosidic bridge or to the cell membrane (lipoteichoic acids, LTAs) via a glycolipid anchor (Fig. 2A) (Neuhaus and Baddiley, 2003). To this backbone, a number of substituents can be linked, among them D-alanine, which can be coupled by an ester bond to free hydroxyl groups of the TA backbone or in some cases to glycosidic substituents (Wicken and Baddiley, 1963; Sadovskaya et al., 2004; Sánchez Carballo et al., 2010). However, it must be noted that D-alanylation is not a general characteristic of TAs, and it is apparently limited to Firmicutes (Neuhaus and Baddiley, 2003). The degree of D-alanylation is highly variable and depends on strain background and growth conditions (Perego et al., 1995; Neuhaus and Baddiley, 2003; McCormick et al., 2011).

The synthesis of D-alanyl-LTAs is accomplished by the concerted action of four proteins encoded by the *dltABCD* operon (Perego et al., 1995; Neuhaus et al., 1996). DltA catalyzes the synthesis of D-alanyl-AMP from D-alanine and ATP and subsequently transfers this intermediary compound to the D-alanyl carrier protein DltC ((Neuhaus and Baddiley, 2003) and references therein). The role of proteins DltB and DltC remain to be determined. DltB is predicted to possess 12 membrane-spanning domains (Neuhaus et al., 1996) and the hydropathy profile also predicts that DltD is anchored to the cell membrane by an N-terminal hydrophobic domain (Debabov et al., 2000).

146 The regulation of *dlt* operon expression is operated by different mechanisms in different
147 species, and usually it is subject to the control of several regulatory systems within the same
148 organism. In *Bacillus subtilis*, *dlt* is part of the regulons of the extracytoplasmic-function sigma
149 factors σ^X (Cao and Helmann, 2004; Kingston et al., 2013), σ^V (Guariglia-Oropeza and Helmann,
150 2011) and the two-component system (TCS) YxdJK (Joseph et al., 2004). In staphylococci, the
151 *dlt* operon is positively regulated by the TCS GraRS (*Staphylococcus aureus*; (Li et al., 2007b)) or
152 its homolog ApsRS (*Staphylococcus epidermidis*; (Li et al., 2007a)) in response to CAMPs, and it
153 is repressed by the TCS ArlRS in response to high extracellular concentrations of Mg^{2+}, Ca^{2+} or
154 Na^+ (Koprivnjak et al., 2006). Furthermore, there is evidence indicating that the global
155 regulators Agr (Dunman et al., 2001) and Rot (Saïd-Salim et al., 2003) are also involved in *dlt*
156 regulation in *S. aureus*. In *Lactobacillus casei*, TCS12 regulates the expression of *dlt*, but
157 induction of *dlt* expression in response to nisin was observed in TCS12-defective mutants,
158 indicating that additional regulatory mechanisms also operate in this organism (Revilla-
159 Guarinos et al., 2013).
160 Studies of *dlt* mutants have shown that D-alanylation of TAs has a wide range of physiological
161 consequences in different bacteria as well as in their interactions with other organisms
162 (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008; Swoboda et al., 2010). This
163 review will only focus on the important role of D-alanylation for the resistance against CAMPs
164 (Fig. 2), as documented by numerous studies (Davie and Brock, 1966; Peschel et al., 1999; Boyd
165 et al., 2000; Abachin et al., 2002; Poyart et al., 2003; Kristian et al., 2005; Fabretti et al., 2006;
166 Kovács et al., 2006; Saar-Dover et al., 2012; Revilla-Guarinos et al., 2013). These observations
167 have been explained by postulating that D-alanylation of TAs would diminish the electrostatic
168 attraction between CAMPs and the cell envelope by reducing the net charge of the cell wall
169 (Fig. 2B) (Peschel et al., 1999; Neuhaus and Baddiley, 2003; Peschel and Sahl, 2006; Swoboda
170 et al., 2010; Anaya-López et al., 2013). This model is in accordance with different experimental
171 observations demonstrating that a lack of alanylation leads to increased binding of several

positively charged molecules such as Mg^{2+} (Heptinstall et al., 1970) or cytochrome *c* (Cyt *c*) (Wecke et al., 1997; Peschel et al., 1999; Kristian et al., 2005; Saar-Dover et al., 2012; Revilla-Guarinos et al., 2013) and also the CAMPs gallidermin (Peschel et al., 1999) and vancomycin (Peschel et al., 2000).

While this model (Fig. 2B) is generally accepted, a number of recent observations have challenged it. A *dltA* mutant of *Streptococcus agalactiae* was shown to bind three times more Cyt *c* than the wild-type strain. However, no significant differences in binding of a number of CAMPs were detected, indicating that different interactions account for the binding of Cyt *c* and the binding of CAMPs (Saar-Dover et al., 2012). A direct estimation of the net electric charge of *Lactococcus lactis* cells by electrophoretic mobility measurements detected no significant difference in global cell charge between the wild-type strain and a *dltD*-defective mutant (Giaouris et al., 2008). This observation is in accordance with results from similar experiments on *L. casei* in our own laboratory (unpublished results). The estimation of cell electric charge by binding assays relies on the assumption that the interaction between the cell envelope and the ligand is essentially electrostatic and independent of the nature of the ligand. However, the contribution of other interactions should be taken into account. For example, hydrophobic interactions between Cyt *c* and cell membrane lipids have been observed earlier (Rytömaa et al., 1992; Cortese et al., 1995) and might influence the binding affinity for Cyt *c* of the bacterial cell envelope.

Based on these and the following observations, an alternative model (Fig. 2C) was recently proposed that suggests that D-alanylation of TAs leads to structural modifications of the cell wall making it more compact and less permeable and hence restricting the access of CAMPs to the membrane (Saar-Dover et al., 2012). In support, these authors showed that the cell wall of a *S. agalactiae dltA* mutant is less dense and its surface is less rigid than that of the wild-type strain. It was shown that binding of CAMPs to LTA was not significantly different between the two strains; however, access of CAMPs to the membrane was increased in the *dltA*-defective

198 mutant. Furthermore, the authors observed that high NaCl concentration reduced the
199 penetration of CAMPs through the cell wall of the *dltA* strain to restore wild-type behaviour.
200 Previous studies had already noted alterations in the cell wall structure in response to the
201 extent of D-alanylation of TAs. Ou and Marquis observed that removal of D-alanyl esters from
202 TAs of *S. aureus* caused an expansion of the cell wall (Ou and Marquis, 1970). Furthermore, it is
203 well established that TAs play a major role in the structure of the cell wall and that the ionic
204 environment is a determinant in the structural transitions of TAs (Doyle et al., 1974; Pal et al.,
205 1990). Incorporation of D-alanyl residues in TAs would change the ionic environment around
206 TAs, thus modulating the conformational transitions of TAs (Neuhaus and Baddiley, 2003; Saar-
207 Dover et al., 2012). These transitions could account for the structural differences observed
208 between the cell walls of D-alanyl-TAs deficient strains and those of the parental strains. Taken
209 together, this evidence supports the idea that D-alanylation of teichoic acids modifies the
210 electrostatic interactions between TAs leading to a strengthening of the cell wall and an
211 increase of its barrier properties (Fig. 2C). This would impede the access of the usually
212 amphipathic CAMPs to the membrane.
213
214 **CAMP resistance by ABC transporter-mediated antibiotic removal**
215 Recently, a classification scheme for ABC transporters involved in the removal of CAMPs from
216 the cell membrane of Firmicutes based on their predicted domain architectures has been
217 proposed (Gebhard, 2012), which was in accordance with functional characteristics such as
218 transport mechanism and regulation. ABC transporters were classified into five groups, each
219 named after one well-characterized example as SunT-type, NisT-type, LanFEG-type, BceAB-
220 type and BcrAB-type transporters. The first two groups are involved in the export of newly
221 synthetized CAMPs and will not be considered further here. The other three groups of
222 transporters will be described in the following section, highlighting the most striking
223 mechanistic aspects of AMP resistance of each group, and a summary of their main

224 characteristics is presented in Table 1. For reasons of conciseness, only some supporting
225 relevant examples will be discussed. Readers are referred to a recent comprehensive review
226 for further information (Gebhard, 2012).
227 Among the resistance transporters two mechanisms of CAMP detoxification can be
228 distinguished (Fig. 3). For LanFEG and BcrAB-type transporters, the transporter is sufficient for
229 partial resistance but additional proteins help to provide full protection from AMPs. In the case
230 of the BceAB-group, the transporter plays a role in sensing, signaling and detoxification of the
231 AMPs.
232
233 **LanFEG and BcrAB type transporters: playing with partners for higher resistance**
234 Most LanFEG-type transporters are involved in self-protection in lantibiotic producer strains,
235 and recognize only a narrow range of related substrates (Otto et al., 1998; Stein et al., 2003;
236 Gebhard, 2012). BcrAB transporters mediate resistance against bacitracin (Podlesek et al.,
237 1995; Neumüller et al., 2001). LanFEG and BcrAB transporters are composed of two permease
238 subunits with six predicted transmembrane helices, which can be encoded by two separate
239 genes (*lanE* and *lanG* in LanFEG-type) or a single gene (*bcrB* in BcrAB-type). The ATPase
240 subunits are encoded by separate genes in both types of transporters (*lanF* and *bcrA*,
241 respectively) (Gebhard, 2012). Phylogenetic analyses have shown that BcrAB and LanFEG are
242 closely related, and they also share functional characteristics (Gebhard, 2012). Several studies
243 reported that these transporters remove lantibiotics from the cytoplasmic membrane and
244 discharge them to the extracellular medium (Stein et al., 2003; Stein et al., 2005; Okuda et al.,
245 2008) (Fig. 3A, step 3). It remains unclear, however, how cells prevent CAMPs from binding
246 again to the cytoplasmic membrane. In this regard, the high degree of co-occurrence of
247 LanFEG-type transporters with LanI or LanH immunity proteins (78%), and of BcrAB-type
248 transporters with UppP (undecaprenyl pyrophosphate phosphatase)-encoding genes (77%)
249 should be noted (Gebhard, 2012). To date, conflicting data is reported on whether

transporters and immunity proteins act cooperatively or independently of each other to confer resistance. An independent action has been proposed for the nisin resistance system of *Lactococcus lactis*, constituted by the immunity protein NisI and the transporter NisFEG and for the SpaI-SpaFEG system of *Bacillus subtilis*, which provides self-protection against subtilin (Stein et al., 2003; Stein et al., 2005). Other studies suggested cooperativity between NisI and NisFEG (Ra et al., 1999; Takala et al., 2004; Takala and Saris, 2006), or between the nukacin ISK-1 immunity protein NukH and NukFEG (Okuda et al., 2008). It is attractive to postulate a concerted action of transporters and immunity proteins, which might explain the mechanism of resistance: the transporter would remove cell membrane-associated CAMPs and release them to the external media while immunity proteins would bind and sequester the CAMPs, thus avoiding re-association with the bacterial surface (Fig. 3A, steps 1 and 2) (Takala et al., 2004).

BcrAB-type transporters are often encoded in an operon with a UppP encoding gene (Gebhard, 2012). It is therefore likely that the bacitracin resistance mechanism of the transporter is tightly linked to UppP activity (Fig. 3A, steps 3 and 4). In fact, it has been shown that increasing UppP activity confers increased resistance to bacitracin (Bernard et al., 2005; Shaaly et al., 2013), whereas its inactivation led to increased sensitivity (Cao and Helmann, 2002; Shaaly et al., 2013). Therefore, maximal protection is most likely ensured when transporter and UppP act concertedly (Podlesek et al., 1995).

The efflux mechanism used by these transporters still awaits elucidation although the hydrophobic vacuum-cleaner model, originally proposed for the eukaryotic P-glycoprotein, a multidrug ABC transporter (Raviv et al., 1990), currently receives major acceptance. This model hypothesizes that the target compounds enter the transporter binding sites directly from the membrane and are released to the extracellular medium. Subsequent studies demonstrated that the P-glycoprotein binds its substrates within the inner leaflet of the membrane and releases them to the extracellular medium (Shapiro et al., 1997; Shapiro and Ling, 1998) as

postulated by the hydrophobic vacuum cleaner model. In the same way, transport from the inner leaflet to the extracellular medium was demonstrated for the *L. lactis* multidrug resistance ABC transporter LmrA (Bolhuis et al., 1996). However, it remains to be seen if such a mechanism is directly applicable to the CAMP transporters discussed here, whose substrates are most likely located in the outer leaflet of the membrane.

BceAB-type transporters: sensors, triggers and detoxification pumps with a broad range of substrates

BceAB-type transporters mediate resistance to CAMPs but are usually not associated with biosynthetic loci. In contrast to BcrAB and most LanFEG-type transporters, BceAB-type transporters display a broader substrate range but can also distinguish between structurally similar substrates (Table 1) (Gebhard and Mascher, 2011; Gebhard, 2012). For example, the *B. subtilis* PsdAB transporter is able to transport the lantibiotic actagardine but not the similar one mersacidin. At the same time, PsdAB also transports the lipodepsipeptide enduracidin but not the structurally similar ramoplanin (Staroń et al., 2011). The molecular mechanism behind this characteristic is still unclear.

The most noticeable feature of BceAB-type transporters is their frequent genetic and functional association with BceRS-type TCS (Fig. 3B) (Joseph et al., 2002; Mascher, 2006; Dintner et al., 2011). A phylogenetic analysis demonstrated the coevolution of these transporters and TCS in Firmicutes, supporting the functional link between them (Dintner et al., 2011). These Bce-like modules, named after the bacitracin resistance module BceRSAB of *Bacillus subtilis* (Mascher et al., 2003; Ohki et al., 2003), are antimicrobial peptide detoxification systems in which the transporter plays a dual role: it mediates AMP resistance/detoxification and is also required for AMP sensing (Rietkötter et al., 2008). The ABC transporter BceAB detects the stimulus, i.e. presence of bacitracin, and transfers the signal to the histidine kinase (HK) BceS, which does not function as a direct sensor but rather

as a signal transfer relay to BceR. Activation of the response regulator BceR then induces the expression of *bceAB* and thus ensures resistance. Experimental evidence from a number of homologous systems from *B. subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Lactobacillus casei* has confirmed such a signaling pathway as a general characteristic of Bce-type modules (Rietkötter et al., 2008; Ouyang et al., 2010; Hiron et al., 2011; Staroń et al., 2011; Falord et al., 2012; Revilla-Guarinos et al., 2013). Interestingly, some BceAB-like transporters appear to have developed specified functions. While some display the dual role described above, others function only as a sensor or only as a resistance pump (Fig. 3B). In the latter case, two transporters and one TCS are required to constitute a functional Bce-like module, as will be described in the following paragraphs.

Sensing transporters (Fig. 3B, model 5) detect the presence of a CAMP and transfer the signal to their cognate HKs but do not *c*onfer resistance. However, it is worth noting that ATP hydrolysis by the transporter is still required for the signaling process (Rietkötter et al., 2008; Hiron et al., 2011). It has been suggested that transport by these transporters takes place at a low rate that is enough for signaling the presence of the antibiotic to the partner HK, but not sufficient for conferring resistance to it (Gebhard and Mascher, 2011). A characteristic feature of Bce-like modules harbouring a sensing ABC is that they usually control an extended regulon that includes ABC transporters (the sensing and/or associated resistance transporters), genes involved in the cell envelope stress response like *dltABCD* and *mprF* and genes for cell wall biosynthesis (Fig. 3B, model 5). In the Aps/GraRS-VraFG system, VraFG is the sensing transporter, and resistance involves expression of the *dlt*-operon and *mprF*, which together with VraFG are under transcriptional control of the TCS Aps/GraRS (Li et al., 2007b; Meehl et al., 2007; Falord et al., 2011; Falord et al., 2012). Another complex Bce-like regulatory network of AMP detoxification modules was recently described in *L. casei* BL23 (Revilla-Guarinos et al., 2013). Module 12 of this strain was shown to be a sensory system controlling CAMPs resistance. ABC12 is the sensory transporter that communicates with TCS12, which in turn

ensures the expression of *dltABCD*, *mprF*, and an additional "orphan" BceAB-type ABC transporter that is located in a different position of the chromosome.

Dedicated resistance transporters (Fig. 3B, model 6) mediate the actual resistance to the antibiotic, but are not involved in peptide sensing and signaling. They are usually controlled by a not genetically associated BceRS-type TCS, which is typically encoded together with a sensory transporter. A characterized example is the VraDE transporter of *S. aureus*, which mediates resistance to CAMPs and is under control of the BraRSDE module, where BraRS is the TCS and BraDE the sensory transporter (Hiron et al., 2011).

The third group of BceAB-type systems consists of ABC transporters with a dual function: they are involved in substrate sensing and signaling and also confer resistance to it. Hence, these transporters regulate their own expression in response to AMPs via BceRS-like TCS (Fig. 3B, model 7) (Rietkötter et al., 2008). Once the inducing compound is removed, the system switches off. An example of these systems is module 09 of *L. casei*. ABC09 mediates resistance to bacitracin, nisin, plectasin, and subtilin (Revilla-Guarinos et al., 2013). Its expression is induced in a concentration dependent manner by nisin through the cognate TCS09, which depends on ABC09 for its activation. Accordingly, module 09 is a stand-alone resistance module where ABC09 senses the target CAMPs and transfer the signal to TCS09, resulting in the induction of the expression of ABC09, which confers the resistance (Revilla-Guarinos et al., 2013). The same is true for at least two out of the three *B. subtilis* Bce-type resistance modules, which also possess transporters with a dual function. BceRSAB is the most effective bacitracin resistance system (Mascher et al., 2003; Ohki et al., 2003), but it also confers resistance to mersacidin, actagardine and plectasin (Staroń et al., 2011). The paralogous system PsdRSAB is induced by enduracidin, actagardine, gallidermin, nisin and subtilin, and it mediates resistance to all of its inducers excepting actagardine (Staroń et al., 2011). Both systems are stand-alone detoxification modules (Ohki et al., 2003; Rietkötter et al., 2008; Staroń et al., 2011).

354 Although the role of Bce-type resistance modules in the regulation of transcription has been
355 thoroughly studied, details of the mechanism of transport and signal transduction have not
356 been completely determined.
357
358 **Open questions and concluding remarks**
359 Significant progress has been made in the last years to understand the major systems that
360 confer CAMP resistance in Firmicutes, both with regard to the role of D-alanylation and the
361 function of designated ABC transporters. Nevertheless, the studies summarized above have
362 also led to a number of open questions that still need to be addressed in order to provide
363 mechanistic insights into how those systems work.
364 The identification of a potential second mechanism by which D-alanylation of TAs affects
365 CAMP sensitivity raises the question, whether electrostatic and steric hindrance are mutually
366 exclusive concepts or whether both contribute to CAMP resistance (Fig. 2). The evidence for
367 both mechanisms argues in favor of the latter, but further studies will be necessary to answer
368 these questions.
369 In the transporter-mediated resistance, both the mechanism of substrate binding and, in the
370 case of BceAB-like systems, the direction of transport have not been determined so far (Table
371 1). BceB-like permeases are membrane proteins with ten predicted transmembrane helices
372 and a large extracytoplasmic domain (ECD) of approximately 200 amino acids. While
373 phylogenetic analyses of the transmembrane regions of BceB-like transport permeases
374 showed good sequence conservation at the amino acid level, the ECD regions did not (Dintner
375 et al., 2011). This high degree of variability of the ECD agrees with the proposal that this region
376 of the permease contains the substrate binding domain of the transporter (Rietkötter et al.,
377 2008), and that the high degree of variability reflects the wide range of CAMPs to which they
378 confer resistance (Dintner et al., 2011). Some experimental results support this idea. The *S.*
379 *aureus* ABC transporter VraDE confers bacitracin resistance while VraFG is involved in

resistance to colistin. Domain-swapping studies showed that a transporter with a chimeric VraG permease harbouring the ECD of VraE, *vraFG*^{*vraE}, restored bacitracin resistance in a *ΔvraDE* mutant but was not able to restore colistin resistance in a *ΔvraFG* mutant strain (Hiron et al., 2011).

Moreover, the exact molecular mechanism by means of which the signal information is transferred from the transporter to the HK is also not known yet. It has been proposed that BceAB-type transporters might function as importers so that detection by the cognate HKs and CAMP inactivation would occur in the cytoplasm (Rietkötter et al., 2008; Hiron et al., 2011). However, the identification of mutations in BceB that significantly decreased signaling activity while retaining bacitracin resistance seems to rule out this hypothesis, at least for the BceRSAB module (Kallenberg et al., 2013). A second hypothesis postulates that the transporter binds the substrate and presents it to the HK, which would then only recognize it in complex with the transporter (Schrecke et al., 2012). In this case signal detection by the HK might occur through the short extracytoplasmatic loop of the HK. This idea is supported by results obtained with the homologous HKs GraS of *S. aureus* and ApsS of *S. epidermidis*. These two proteins show an overall 70% similarity, which is reduced to 33% for the extracellular loop. ApsS responds to hBD3 whereas GraS does not. However, a hybrid GraS with the ApsS extracellular loop responds to hBD3 (Li et al., 2007b). But this hypothesis does not explain why ATP hydrolysis by the transporter is required for signal transfer, since substrate binding should be ATP-independent. The third hypothesis postulates that signal transfer occurs by direct protein-protein contact between the ABC transporter and the HK, where a conformational change in the transporter due to substrate binding and transport could activate the HK. This hypothesis is supported by results obtained by two-hybrid assays carried out with the GraXSR-VraFG system of *S. aureus* (Falord et al., 2012) and the BceRSAB of *B. subtilis* (Kallenberg et al., 2013) which revealed interactions between HKs and cognate ABC transporters.

Different strategies for CAMP resistance in Firmicutes have been reviewed in this work, which, while being very distinct from one another, all serve the same purpose: to enhance bacterial survival in competitive environments. The recently proposed electrostatic-steric hindrance model for the Dlt-mediated resistance as well as the many unanswered questions regarding ABC transporters, highlight the complexity of this subject. Given the significant amount of progress made in recent years and the diversity of different organisms and experimental approaches currently applied to study both mechanisms of CAMP resistance, one can be optimistic that some, if not most of the above questions will be addressed and eventually solved.

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Figure legends

Fig. 1. A. Structural and compositional diversity of antimicrobial peptides. Schematic representations of the structures of nisin, bacitracin, ramoplanin and protegrin. The amino acids are represented by labeled gray circles. Positively and negatively charged amino acids at neutral pH are highlighted in red and white, respectively. Abu, aminobutyric acid; Chp, L-3-chloro-4-hydroxyphenylglycine; Dha, didehydroalanine; Dhb, didehydrobutyrine; HAsn, β-hydroxyasparagine; D-Hpg, D-hydroxyphenylglycine; L-Hpg, L-hydroxyphenylglycine; Man, mannose; Orn, D-ornithine; aThr, D-*allo*-threonine.
B. Schematic representation of peptidoglycan biosynthesis and its inhibition by CAMPs. Important steps in cell wall biosynthesis are depicted, and their cellular location is indicated on the left. CW, cell wall; CM, cytoplasmic membrane; NAG, N-acetyl-glucosamine; NAM, N-acetyl-muramic acid; UP, undecaprenyl-phosphate; UPP, undecaprenyl-pyrophosphate. Amino acids are symbolized by small grey circles. Lipid II consists of the NAG/NAM-pentapeptide building block, covalently linked to the lipid carrier molecule UP via a pyrophosphate ester bridge. The steps of cell envelope biosynthesis linked to UP are referred to as "Lipid II cycle". CAMPs are placed next to the step they inhibit.

Fig. 2. Models of the effect of changes in the bacterial cell surface in CAMPs resistance. CM, cytoplasmic membrane; CW, cell wall; WTA, wall teichoic acids; LTA, lipoteichoic acids. CAMPs are depicted as red stars.

A, cell envelope in the absence of D-alanylation of TAs and L-lysinilation of membrane phospholipids. Local concentration of CAMPs is increased presumably by electrostatic interactions with the cell envelope. CAMPs can reach the cell membrane and interact with their targets.

B, electrostatic hindrance model for CAMP resistance. D-alanylation of TAs and L-lysinylation of membrane phospholipids decrease the net negative charge of the cell envelope and the local concentration of CAMPs.

C, electrostatic and steric hindrance model. D-alanylation of TAs modifies the cell wall structure making it less permeable to CAMPs.

Fig. 3. Schematic representation of the postulated models of action of ABC transporters conferring CAMP resistance.

A, LanFEG and BcrAB transporters. Transporters are shown in green, and ATP-hydrolysis and substrate translocation are indicated by black solid and dashed arrows, respectively. CAMPs are shown as red stars. 1) Transport assisted by NukH-type immunity proteins. 2) Binding of CAMPs by NisI-type immunity proteins. 3) Hydrophobic vacuum-cleaner model of efflux mechanism. 4) CAMPs (bacitracin) bind to the pyrophosphate group of UPP preventing its dephosphorylation by undecaprenyl pyrophosphate phosphatase (UppP; pink pentagon); UPP and UP molecules are shown schematically and dephosphorylation is indicated by a black arrow. IM, immunity protein.

B, BceAB-type transporters. 5) Sensing, 6) resistance and 7) dual function transport systems. Signaling between the transporters (green) and the TCS (blue) is indicated by a double-headed black arrow. Phosphotransfer within TCS and gene activation are indicated by black arrows, and the increased expression of transporter genes is indicated by straight dotted arrows. The positions of promoters relative to genes were chosen arbitrarily. Likely dimerization of BceB-type permease subunits is not shown for reasons of simplicity. HK, histidine kinase; RR, response regulator.

Table 1: Summary of ABC transporters main characteristics. Based on (Gebhard, 2012).

	LanFEG	BcrAB[a]	BceAB
Domain architecture	Permeases of 200–250 aa[b] and six TM[b] helices each	Permeases of approximately 230 aa with six predicted TM helices	Permease of approximately 650 aa and 10 TM helices, with a large – approx. 200 aa-extracellular domain located between helices VII and VIII
Direction of substrate transport	Export (the lantibiotic is removed from the cytoplasmic membrane to the culture supernatant)[c]	Unknown (export postulated)[d]	Unknown. Import suggested, followed by cytoplasmic enzymatic inactivation of the CAMP through degradation[e]
Associated proteins	Immunity proteins: LanI-type proteins (tethered to the membrane surface via an N-terminal lipoprotein anchor) and LanH-type proteins (contain three TM helices with the N-terminus located intracellularly)	Undecaprenyl-pyrophosphatase (UppP)	BceRS-like TCS[b]
Regulation[d]	Mostly regulated by a TCS with prototypical periplasmic sensing HK[b,f] and OmpR family RR[b]. Others by XRE family transcriptional regulators	Mostly regulated by a TCS with IM-HK[b, f] and OmpR family RR. Others by XRE transcriptional regulators	BceRS-like TCS with IM-HK and OmpR family RR. Transporter regulating its own expression in response to CAMPs (see text for details)
Physiological role	Mostly involved in self-protection of lantibiotic producing strains (some are genetically associated with lantibiotic biosynthesis genes). Rarely, AMP resistance in non-producing strains.	Resistance against the cyclic AMP bacitracin in producing (self-protection) and non-producing strains	AMP resistance in non-producing strains
Substrates	Lantibiotics (nis, gall, epi, nuk, sub, etc.)[g] and dipeptide lantibiotics (lact)[g]	Cyclic AMP: bac[g]	Lantibiotics (nis, sub, gall, mer)[g], cyclic AMPs (bac), lipodepsipeptides (end)[g], glycopeptides (van, tei)[g], peptides from the immune system of higher organisms like

cathelicidines (LL-37, ind, ovi)[g], and defensins (hBD3, bre, ple)[g]

[a] For simplicity only the BcrAB transporters are included. The reader is referred to Gebhard 2012 (Gebhard, 2012) for additional information on YydIJ.

[b] aa*:* amino acids; TM*:* transmembrane; TCS*:* two component systems; HK*:* Histidine kinase; RR*:* Response regulator; IM-HK*:* intramembrane-sensing histidine kinase.

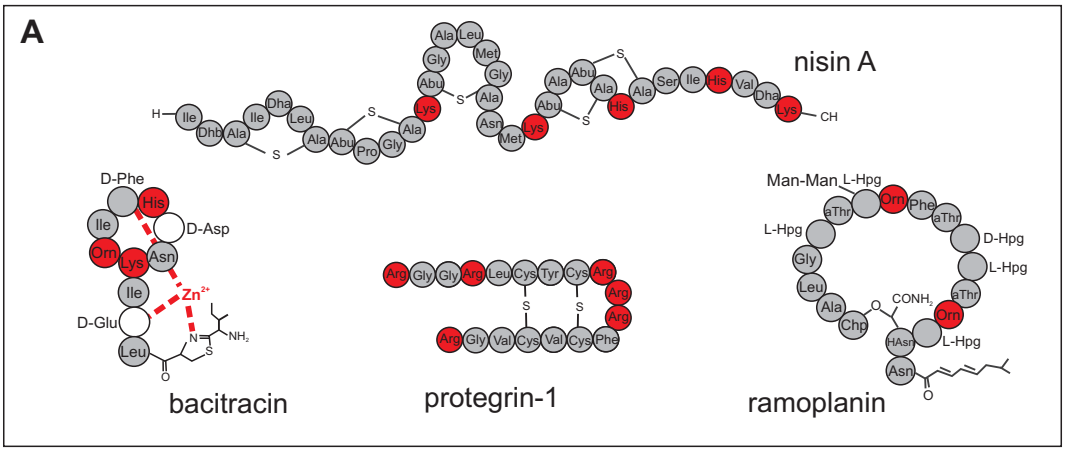
[c] (Otto et al., 1998; Stein et al., 2003; Okuda et al., 2008)

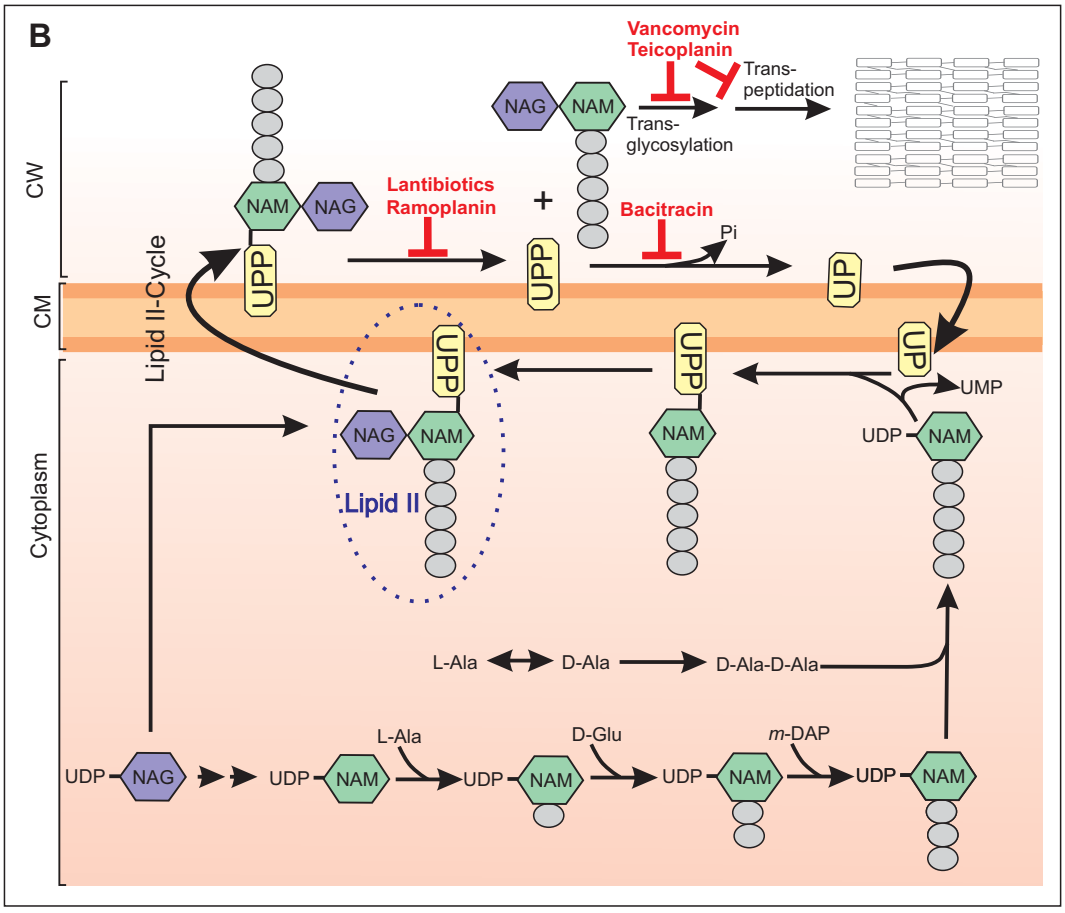
[d] (Gebhard, 2012)

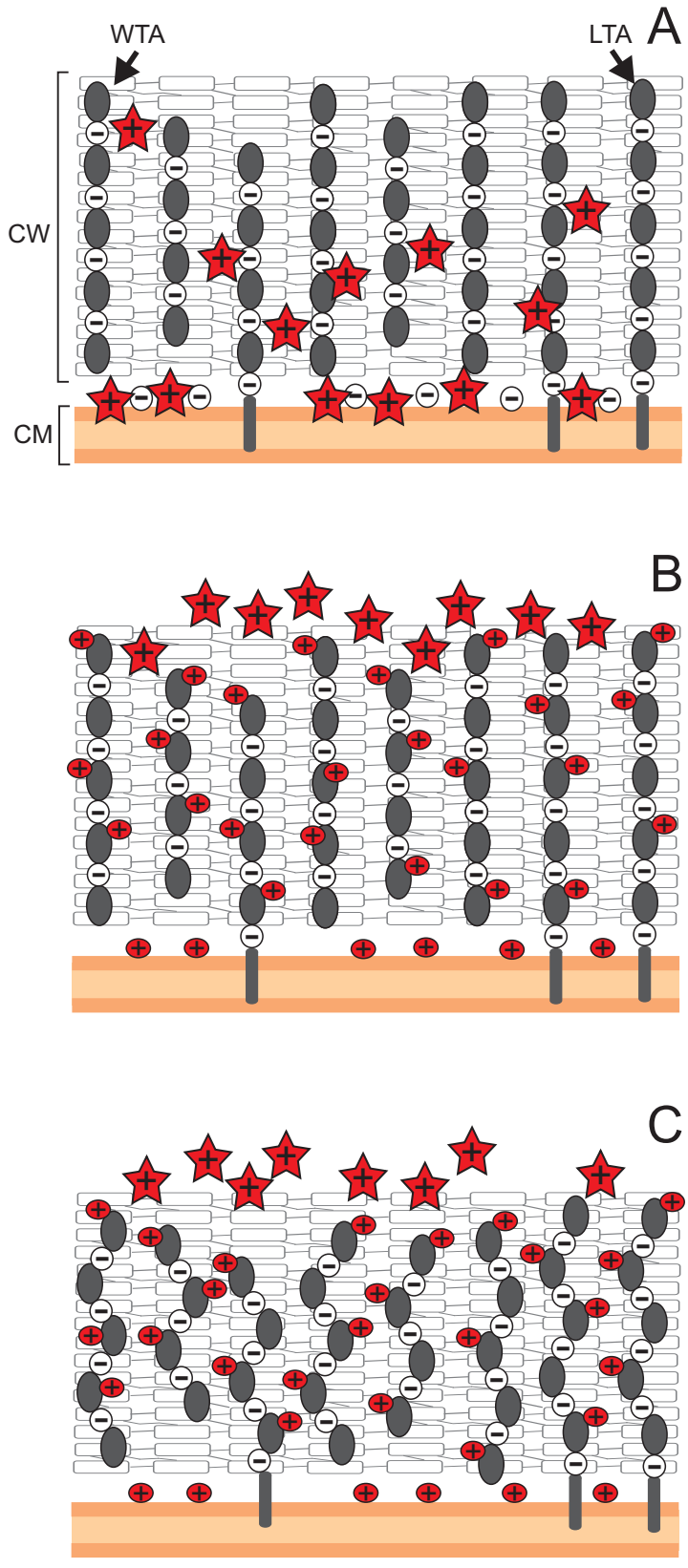
[e] (Rietkötter et al., 2008; Hiron et al., 2011)

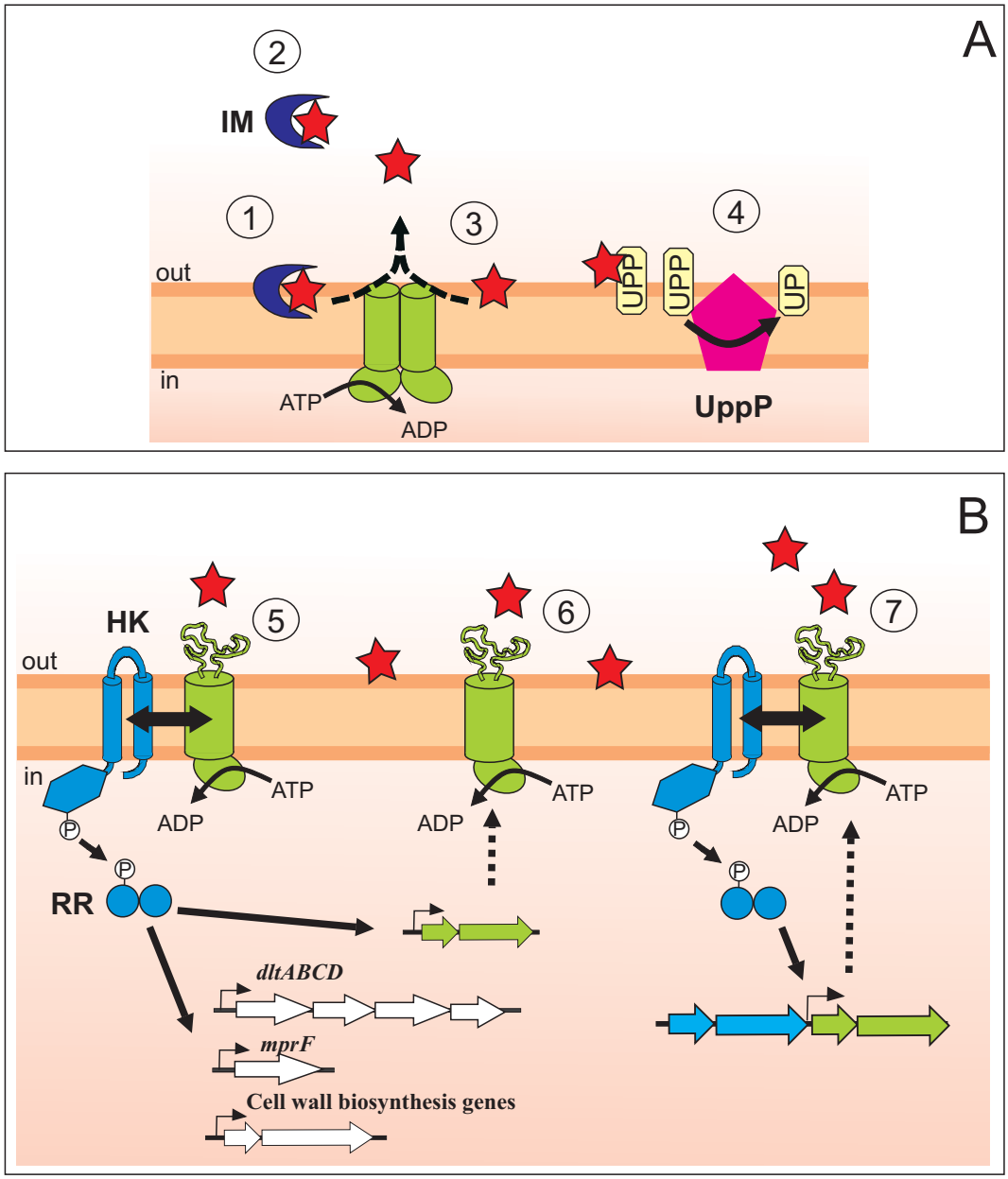
[f] (Mascher et al., 2006).

[g] *act*: actagardine; *bac*: bacitracin; *bre*: brevinin; *end*: enduracidin; *epi*: epidermin; *gall*: gallidermin; *ind*: indolicidin; *lact*: lacticin 3147; *mer*: mersacidin; *nis*: nisin; *nuk*: nukacin; *ovi*: ovispirin; *ple*: plectasin; *sub*: subtilin; *tei*: teicoplanin; *van*: vancomycin.


A
nisin A
bacitracin
protegrin-1
ramoplanin


B
Vancomycin
Teicoplanin
Trans-
peptidation
Trans-
glycosylation
Lantibiotics
Ramoplanin
Bacitracin
Pi
UMP
CW
CM
Cytoplasm
Lipid II-Cycle
Lipid II
UPP
UP
NAG
NAM
UDP
L-Ala
D-Ala
D-Ala-D-Ala
D-Glu
m-DAP


A
WTA
LTA
CW
CM
B
C


A
2
IM
1
3
4
out
in
ATP
ADP
UPP
UPP
UP
UppP
B
HK
5
6
7
out
in
ATP
ADP
ATP
ADP
ATP
ADP
P
P
RR
dltABCD
mprF
Cell wall biosynthesis genes